Hemoglobin Variant HbG-Coushatta (β -22 Glu $\rightarrow$ Ala) Found by Dissociation of Blood Glucose from Values of HbA_{1c} Measured by HPLC

Koichi OGAWA, Tomoko BANDO, Maki OGAWA, Ayako MIYAZAKI*,
Toyohumi NAKANISHI* and Akira SHIMIZU*

Abstract

Objective Unexpectedly low values of HbA_{1c} measured by HPLC compared with their blood glucose levels were found in three related persons. We investigated whether this discrepancy was due to abnormal hemoglobin.

Patients and Methods HbA_{1c} was measured by latex agglutination and ordinary HPLC. For further examination, a hemoglobin specimen from the 82-year-old female case was prepared and analyzed by PolyCAT A chromatography, ESI/MS and MS/MS. The HbA_{1c} levels of the three cases measured by HPLC were lower than those measured by latex agglutination. The elution profiles on HPLC of the three gave an unusual peak between HbA_{1c} and HbA₀.

Results PolyCAT A chromatography revealed two additional peaks which were not present in normal hemolysates. These peaks were revealed to correspond to abnormalities of HbA₀ and HbA_{1c}. The amounts of HbA₀ and HbX were almost the same (1 : 0.85) and their glycation ratios were almost equal (5.2% and 5.9%). ESI/MS showed that the woman's intact globin contained an abnormal β^x -chain in addition to the normal α^A - and β^A -chains. The molecular weight of this abnormal β^x -chain was 58 Da lower than that of the normal β^A -chain. Glutamate at the 22nd amino acid residue of the β^A -chain was replaced by alanine in the abnormal β^x -chain. This variant of Hb was revealed to be the same as HbG-Coushatta (β -22 Glu $\rightarrow$ Ala) from the library of variant Hb. Family studies showed that the variant was inherited as a dominant trait.

Conclusion The dissociation was due to underestimation of HbA_{1c} in the measurement by HPLC which excluded glycated variant Hb.

(Internal Medicine 42: 781–787, 2003)

Key words: HbA_{1c}, variant hemoglobin, HbG-Coushatta, family study, tandem mass spectrometry

Introduction

Glycohemoglobin (HbA_{1c}), which is defined as hemoglobin (Hb) irreversibly glycosylated at the N-terminal valine of the β -chain, serves as an indicator for the diabetic state and for the control of diabetes mellitus. HbA_{1c} is measured by various methods including latex agglutination, and high performance liquid chromatography (HPLC). Dissociation of HbA_{1c} values obtained by HPLC from blood glucose levels was recognized in 3 of 340 persons on routine physical examinations in our clinic in 2001. The HbA_{1c} values of these persons were lower than those obtained by latex agglutination. An unusual peak was observed between the HbA_{1c} and HbA₀ peaks in the elution profile on HPLC. The dissociation, low HbA_{1c} and unusual peak suggest that the samples might have contained variant Hb (1,2). Here, we report the analysis and identification of the variant Hb.

Materials and Methods

1) Determination of HbA_{1c} for routine health care examination.

Values of HbA_{1c} were measured by latex agglutination immunoassay or ordinary HPLC.

2) Hemoglobin.

An Hb specimen from an 82-year-old Japanese female, whose elution profile of ordinary HPLC gave an unusual peak, was subjected to structural analysis. Her laboratory data were as shown in Table 1. An isopropanol stability test of the hemolysate was negative. All clinical studies were conducted in accordance with the principles of the

Table 1. Hematologic and Blood Chemical Data

	Case 1	Case 2	Case 3
Age	82	55	52
Sex	female	female	female
Height (cm)	152	150	154.1
Weight (kg)	51.5	50	57
Variable			
Red cells ($\times 10^4$ per mm^3)	434	489	475
Hemoglobin (g/dl)	13.3	15	14.7
Hematocrit (%)	41.6	44.2	42.5
Reticulocyte count (%)*	7	14	22
White cells (per mm^3)	4,700	7,900	4,400
Aspartate aminotransferase (U/l)	14	23	27
Alanine aminotransferase (U/l)	10	20	48
Urea nitrogen (mg/dl)	12.3	15.9	10.3
Creatinine (mg/dl)	0.5	0.5	0.4
Total bilirubin (mg/dl)	0.7	0.4	0.5
Total serum protein (g/dl)	6.7	7.4	7.1
Fasting blood glucose (mg/dl)	84	85	110
HbA _{1c} (%)	2.3	2.5	2.9
Glycoalbumin (%)**	14.3	13.8	17.5

Normal range; Reticulocyte count*: 2~20%, Glycoalbumin**: 12.0~16.3%.

Figure 1. Elution patterns on routine HPLC of typical normal persons and cases 1 to 3. SA_{1c} represents stable HbA_{1c}, P₀₁ undetermined peak, A₀ hemoglobin. Amounts of each peak are indicated above each elution profile.

Declaration of Helsinki.

3) Further estimation of variant Hb.

For further estimation of the abnormal peak, high resolution weak cation-exchange column chromatography (4.6×200 mm) using PolyCAT A (PolyLC Inc., Columbia, MD, USA) was performed as described (3).

4) Electrospray ionization mass spectrometry (ESI/MS) using TSQ-7000 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (Finnigan MAT, San Jose, CA, USA), and tandem mass spectrometry (MS/MS) using HPLC of Alliance2690 (Waters Corporation, Milford, MA, USA) and XTerra™ MS C18 column (2.1×150 mm), 5 mm (Waters Corporation) were carried out. Preparation of globin and measurement of mass spectra of intact globin were performed as reported (4, 5).

After cleavage of the globin with TPCK-trypsin, analysis of the peptides by ESI/MS and MS/MS connected to reverse phase HPLC was performed for sequence determination. This process included collision-induced dissociation (CID). These methods were carried out as previously reported (4, 5).

Results

Dissociation of HbA_{1c} measured by ordinary HPLC from blood glucose levels

Dissociation of HbA_{1c} from blood glucose was observed in three related individuals on routine health care examination. HbA_{1c} was measured by the latex agglutination method from 1997 to 1999, and by ordinary HPLC after 2000. HbA_{1c} levels measured by HPLC were lower than those measured by latex agglutination in all three cases.

The elution profile of ordinary HPLC of cases 1 to 3 gave an unusual peak between HbA_{1c} and HbA₀ as shown in Fig. 1.

This unusual peak is likely to be that of glycated variant Hb, which is a cause of low HbA_{1c}. Unglycated variant Hb is thought to be eluted in the HbA₀ fraction in these experimental conditions. Profiles and laboratory data of these cases are shown in Table 1.

Separation of hemoglobin components in hemolysates from case 1 was performed by high resolution PolyCAT A (Fig. 2). Two additional peaks which were not present in normal hemolysates were found; the peak termed HbX, eluted after the peak of HbA₀, and the peak termed HbX_{1c} which was eluted after the peak of HbA_{1c}. These correspond to the non-glycated variant Hb and glycated variant Hb, respectively. The amounts of HbA₀ and HbX were almost the same (1 : 0.85). The glycation ratios of normal Hb (HbA₀) and variant Hb (HbX) were almost equal, being 5.2% and 5.9%, respectively. These results revealed that the variant Hb was inherited heterozygously.

From the finding of the same levels of glycation in the Hbs, the total glycated Hb levels of the cases were thought to be the sums of SA_{1c} and P₀₁ obtained by ordinary HPLC,

Figure 2. High resolution weak cation-exchange chromatography (PolyCAT A) separation of hemoglobin components in hemolysates from case 1. A₀ and X represent normal Hb and variant Hb, and A_{1c} and X_{1c} indicate glycated Hb. The ratio of A₀ to X was 1 : 0.85. The glycation ratios of A₀ and X were 5.2 and 5.9%, respectively.

being 4.4, 4.6 and 4.9 in cases 1 to 3 respectively. These values obtained by calculation were in good agreement with the values obtained by latex agglutination methods, which are not able to distinguish glycated normal Hb and glycated variant Hb.

Analysis of variant Hb

The intact globin from case 1 was analyzed by ESI/MS. As shown in Fig. 3, the peak of non-glycated α globin was single, whereas the peaks of β globin were double. The abnormal β^x -globin peak was observed at a position of 58Da lower molecular weight than that of normal β^A -globin. The sum of the amounts of β^A and β^x -globin were the same as that of α globin, suggesting heterozygosity. Glycated α^A , glycated β^x and glycated β^A were also observed at the same positions as those with molecular weight with added glucose.

The peptides cleaved by TPCK-trypsin were analyzed by online HPLC-ESI/MS. A peptide of 58Da less than β T-3 was found, and designated as β T^x-3, as shown in Fig. 4.

On analysis using collision-induced dissociation (CID)

Figure 3. Transformed ESI/MS spectrum of the globin from case 1. α^A indicates the normal α -chain, and β^x indicates the β -chain of variant Hb. Glycated peaks were also observed.

mass spectra, both normal βT -3 and abnormal βT^x -3 were found to have a common b series from b_1 to b_4 and a common y series from y_1 to y_8 . The molecular weight of abnormal y_9 and abnormal b_5 were both 58Da less than those of their normal counterparts. The peptides βT -3 (β -chain 18~30) are thought to be constituted from the amino acids proposed in Fig. 5. These results suggested that glutamate at the 22nd amino acid residue of the β -chain was replaced by alanine being 58 Da less than that of glutamate. This variant of Hb was revealed to be the same as HbG-Coushatta [$\beta 22$ (B4) Glu→Ala] from the library of variant Hb (6).

Family studies

An examination on the levels of HbA_{1c} of their family members was carried out. Low values of HbA_{1c}, probably containing variant Hb, were observed in all but two family members as shown in Fig. 6. This strongly suggested that this variant was inherited as a dominant trait.

Discussion

Unexpectedly or unreasonably low values of HbA_{1c} compared to blood glucose or other parameters of diabetes mellitus patients have occasionally been found, since the

Table 2. Comparative Studies by the Latex Agglutination Method and Ordinary HPLC in Assay of HbA_{1c}

Year	case 1			case 2			case 3		
	Blood Glucose*	HbA _{1c} **		Blood Glucose	HbA _{1c}		Blood Glucose	HbA _{1c}	
		Latex	HPLC		Latex	HPLC		Latex	HPLC
1997	83	4.6	nt	84	4.5	nt	97	5.2	nt
1998	97	4.7	nt	118	4.3	nt	107	5	nt
1999	107	4.5	nt	89	4.5	nt	106	5.1	nt
2000	92	nt	3.1	77	nt	2.4	91	nt	3
2001	84	nt	2.3	85	nt	2.5	190	nt	2.9

*: mg/dl, **: %, nt: not tested.

Figure 4. Base ion chromatograms and reconstituted selected ion chromatograms by HPLC-ESI/MS of peptides digested with trypsin. Upper: Base peak ion chromatogram. Middle and Lower: The ion, $m/z=629.5$, is a doubly charged ion of a hypothetical abnormal peptide, named βT^x-3 . The ion, $m/z=658.0$, is a doubly charged ion of normal $\beta T-3$.

Figure 5. CID mass spectra (CID/MS) of a normal peptide (βT -3) and an abnormal peptide (βT^x -3). Comparison of CID mass spectra of a precursor ion of m/z 658.2, the doubly charged precursor ion of normal βT -3 and m/z 629.2, the doubly charged precursor ion of abnormal βT^x -3. The interval of each adjacent y series ion shows the molecular weight of the proposed amino acid. The m/z values of b_5 (499.1) and y_9 (829.4) obtained from βT^x -3 were 58 Da lower than that of the normal counterparts obtained from βT -3. The b-series and y-series ions from the βT -3 region (sequence 18–30 of the β chain) are shown at the top of the figure.

measurement of HbA_{1c} by HPLC was introduced (4, 5). With the specimen containing variant Hb, HbA_{1c} values are underestimated, because the component of HbA_{1c} separated by HPLC does not typically include glycosylated variant Hb. On the other hand, values measured by latex agglutination, that does not distinguish glycosylated variant Hb from glycosylated normal Hb, are apparently reasonable. The HbA_{1c} values measured by latex agglutination were higher than those measured by

HPLC in cases 1 to 3, as shown in Table 2.

The dissociation of blood glucose levels from HbA_{1c} provides a chance to find variant Hb, as described. The variant Hb reported had a structural anomaly at position 22 of the β -chain where an alanyl residue occurred in place of the glutamyl group. This type of variant, HbG-Coushatta, has been found in Koreans, Chinese and North American Indian subjects (7, 8), and in 13 Japanese families. About 860

Figure 6. Pedigree and results of testing for values of HbA_{1c} in the family including cases 1 to 3. Squares represent males, and circles females. Solid squares show males with variant Hb and solid circles show females with variant Hb. The slashed square indicates a deceased person.

different types of variant Hb have been reported to date (6).

The ESI/MS method could be used to clarify the discrepancies between the values measured by ordinary HPLC and those by the latex agglutination method and clinical data, and to obtain more accurate values and detect variant Hb (2).

The estimation of HbA_{1c} is affected by various conditions of subjects; the values of HbA_{1c} from uremic patients are apparently high, because the HbA_{1c} peak on HPLC includes modified Hbs such as carbamyl Hb, and the values of patients with hemolytic anemia are low due to a shortened

erythrocyte survival time. There are also reports that the presence of HbS and HbC are associated with a marked decrease in measurable glycosylation (9, 10). These possibilities were thus tentatively excluded in the present cases.

References

- 1) Nakanishi T, Miyazaki A, Kishikawa M, et al. Quantification of glycosylated hemoglobin by electrospray ionization mass spectrometry. *J Mass Spectrom* **32**: 773–778, 1997.
- 2) Nakanishi T, Miyazaki A, Shimizu A, Yamaguchi A, Nishimura S. Assessment of the effect of hemoglobin variants on routine HbA_{1c} measurements by electrospray ionization mass spectrometry. *Clin Chim Acta* **323**: 89–101, 2002.
- 3) Bisse E, Wieland H. High-performance liquid chromatographic separation of human haemoglobins. Simultaneous determination of foetal and glycosylated haemoglobins. *J Chromatogr* **434**: 95–110, 1988.
- 4) Nakanishi T, Miyazaki A, Kishikawa M, et al. A new hemoglobin variant found during HbA_{1c} measurement: Hb Hokusetsu [β 52 (D3) Asp→Gly]. *Hemoglobin* **22**: 355–371, 1998.
- 5) Nakanishi T, Miyazaki A, Kishikawa M, Shimizu A, Aoki Y, Kikuchi M. Hb Peterborough [β 111 (G13) Val→Phe] in Japan; Rapid identification by ESI/MS using proteolytic digests of oxidized globin. *Hemoglobin* **22**: 23–35, 1998.
- 6) <http://globin.cse.psu.edu/GlobinGeneServer>; April 2002.
- 7) Bowman BH, Barnett DR, Hite R. Hemoglobin G-Coushatta: A beta variant with a delta-like substitution. *Biochem Biophys Res Commun* **26**: 466–470, 1967.
- 8) Blackwell RQ, Ro I-H, Liu C-S, Yang H-J, Wang C-C, Huang JT-H. Hemoglobin variant found in Koreans, Chinese, and North American Indians: $\alpha_2\beta_2^{22\text{Glu}\rightarrow\text{Ala}}$. *Am J Phys Anthropol* **30**: 389–391, 1969.
- 9) Beresford CH, Clegg JB, Weatherall DJ. Haemoglobin Ocho Rio (β 52 (D3) aspartic acid→alanine): A new β -chain variant of haemoglobin A found in combination with haemoglobin S. *J Med Genet* **9**: 151–153, 1972.
- 10) Aleyassine H. Low proportions of glycosylated hemoglobin associated with hemoglobin S and hemoglobin C. *Clin Chem* **25**: 1484–1486, 1979.